Antisense therapy for cancer—the time of truth

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The recent acceleration in the identification and characterisation of new molecular targets for cancer and the limited effectiveness of conventional treatment strategies has focused considerable interest on the development of new types of anticancer agents. These new drugs are hoped to be highly specific for malignant cells with a favorable side-effect profile due to well-defined mechanisms of action. Antisense oligonucleotides are one such class of new agent—they are short, synthetic stretches of DNA which hybridise with specific mRNA strands that correspond to target genes. By binding to the mRNA, the antisense oligonucleotides prevent the sequence of the target gene being converted into a protein, thereby blocking the action of the gene. Several genes known to be important in the regulation of apoptosis, cell growth, metastasis, and angiogenesis, have been validated as molecular targets for antisense therapy. Furthermore, new targets are rapidly being uncovered through coordinated functional genomics and proteomics initiatives. Phosphorothioate oligonucleotides are the current gold standard for antisense therapy; they have acceptable physical and chemical properties and show reasonable resistance to nucleases. Recently, new generations of these phosphorothioate oligonucleotides that contain 2'-modified nucleoside building blocks to enhance RNA binding affinity and decrease indirect toxic effects have been developed. Antisense therapeutics are, after decades of difficulties, finally close to fulfilling their promise in the clinic.

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Recent technological developments that allow for robust data acquisition along with large-scale data-generating programmes, have paved the way for the identification of target genes involved in neoplastic transformation and tumour growth. Identification of the nucleotide sequences of cancer-relevant genes will lead to tailored anticancer agents that lack many of the toxic side-effects displayed by conventional therapeutics.

In this review we discuss the development of exogeneously delivered oligonucleotides for the treatment of cancer and recent progress in clinical application of these treatments. In contrast to the use of plasmid-derived endogenous expression of antisense RNA—which has failed to show activity in vivo because of inefficient plasmid delivery—the antisense oligonucleotide approach (figure 1) has overcome many of barriers to clincial success. Double-stranded, 21-nucleotide, small, interfering RNAs have recently been used to specifically suppress the expression of homologous genes.[1] Available data, however, are too preliminary to conclude that the power of RNA interference

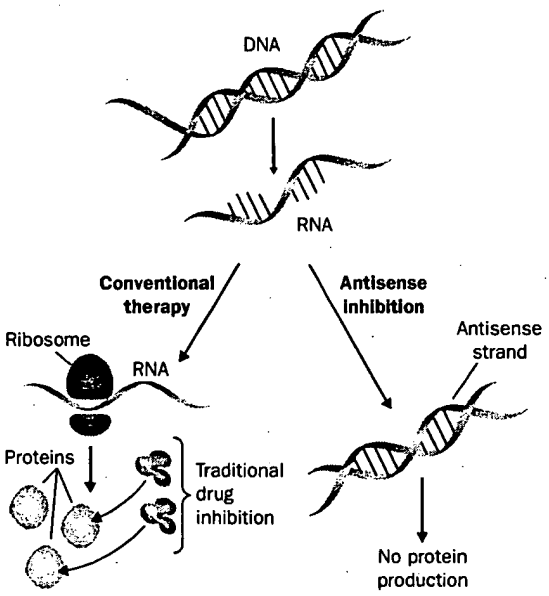

Figure 1. The antisense approach—the basic principle.

can be harnessed for the development of safe and gene-specific therapeutics.

Oligonucleotides have sequences that are complementary to specific strands of RNA. Once delivered into a target cell, the oligonucleotide hybridises with its RNA complement and inhibits expression of the corresponding disease-relevant protein. The idea of oligonucleotide-based antisense therapy is appealling and dates back to the 1960s when Belikova and colleagues[2] proposed that RNA sequences serve as endogeneous inhibitors of gene expression in prokaryotes. In the 1970s, Paterson and colleagues reported that exogeneous, single-stranded nucleic acids inhibit translation of RNA in a cell-free system.[3] 1 year later, Zamecnik and Stephenson did a cell-culture

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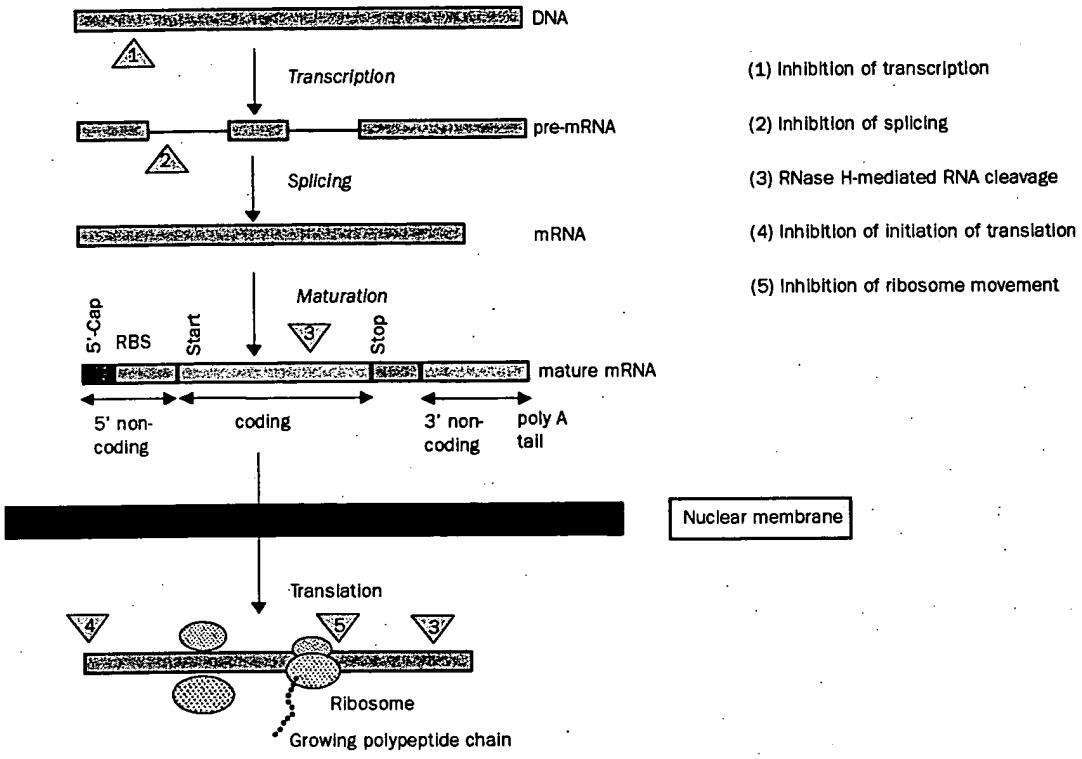

Figure 2. How antisense oligonucleotides work.

experiment which showed that an oligonucleotide complementary to the 3' end of the Rous sarcoma virus could block viral replication in chicken fibroblasts.[4] With the advent of automated DNA synthesis and advances in the field of nucleic-acid chemistry, progress in the use of this technology for target validation and therapy has accelerated considerably.

How antisense oligonucleotides work

Antisense oligonucleotides bind to selected target mRNA molecules by Watson–Crick base pairing, which results in the inhibition of mRNA processing or translation. This inhibition occurs through various mechanisms including prevention of mRNA transport, splicing, and translational arrest (figure 2). The specificity of this approach is based on the estimate that any sequence larger than a minimum number of bases—13 in RNA and 17 in DNA—occurs only once within the human genome. Thus, whereas small-molecule drugs interact with molecular targets through structural recognition, antisense oligonucleotides bind to strands of mRNA on the basis of their sequence. However, effective intracellular delivery remains an important issue for clinical application of antisense oligonucleotides; they have to reach the cytoplasm and finally the nucleus to efficiently access their mRNA target.

Intracellular penetration may occur via energy-dependent endocytosis, which has to be followed by an endosomal or lysosomal escape mechanism, or through a direct cell-membrane permeation process, which is more efficient. Unfortunately, because most oligonucleotides are hydrophilic with an anionic backbone, membrane permeation is low, and simple elimination of anionic charges does not increase this crucial step enough for delivery to be effective. Use of lipophilic transfection reagents such as cationic lipids to form conjugates or complexes, has been widely investigated for delivery of antisense molecules into cells in tissue culture. There have also been several attempts to achieve direct permeation into target cells in vivo. Interestingly, however, in animal models and in patients, all therapeutically active antisense oligonucleotides have been administered in the form of naked compounds, indicating that in intact tissues other mechanisms exist that can act in the same way as cationic carrier lipids.

Inhibition of gene expression is mainly accomplished by sterical hindrance of the target mRNA at the site of ribosomal entry and by recruitment of endogeneous RNase H. Cleavage of target mRNA by RNase H is probably the most important mechanism of antisense action and underlies the activity of all the oligonucleotides successfully tested in clinical trials so far. RNase H is a ubiquitous endonuclease involved in DNA replication, but it may also have other roles in cells. It is found both in the cytoplasm and the nucleus, although the concentration in the nucleus is thought to be greater. RNase H cleaves the RNA strand of a

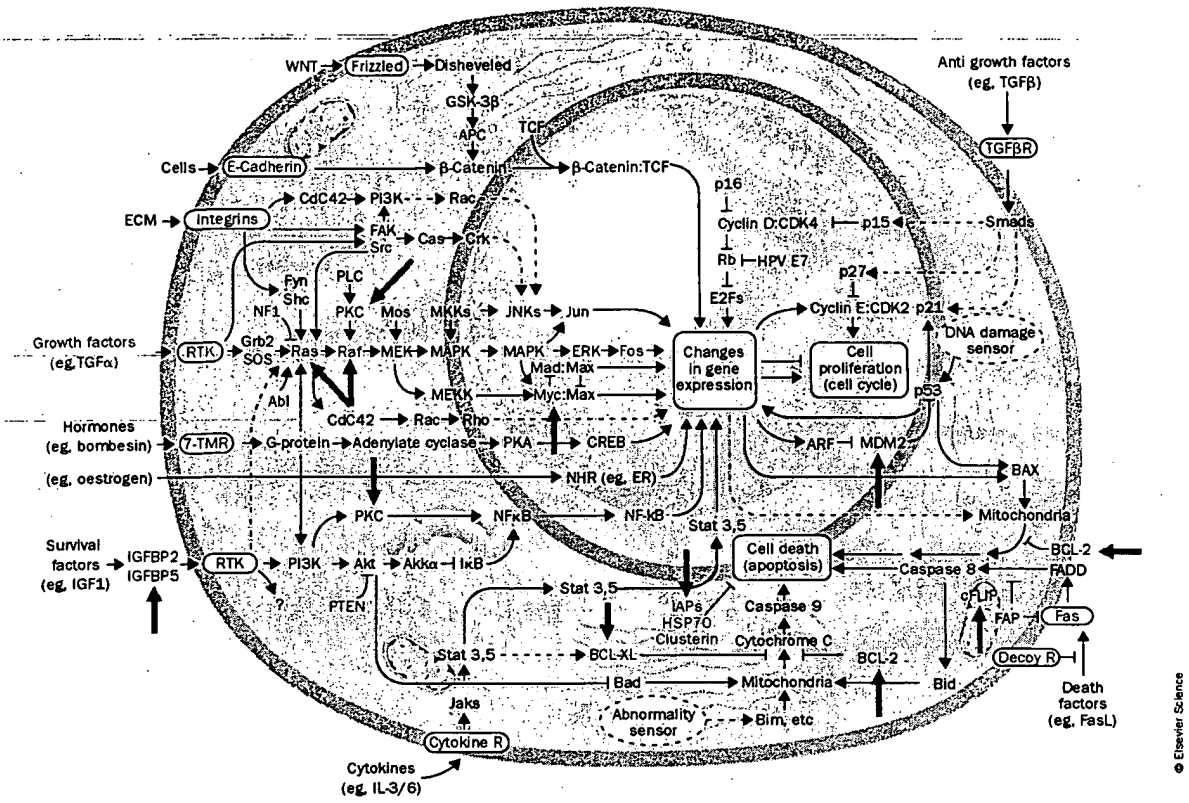

*Figure 3. The emergent integrated circuit of the cell. The blue arrows point at key proteins in pathways that contribute to malignant disease and thus represent promising targets for antisense therapy. Reproduced with modifications from Cell 2000; **100**: 57–70 with permission.*

DNA–RNA heteroduplex. The precise recognition element for the enzyme is unknown, but oligonucleotides with DNA-like properties as short as tetramers seem capable of activating this endonucleolytic process.[5] To what extent these low stringency requirements affect the expression of nonspecific genes (due to irrelevant cleavage) is unclear. In the case of high-affinity oligonucleotides it is likely that a 5 to 7 base homology provides sufficient overlap for RNase H competency.[6] Despite our increasing understanding of how specific hybridisation of antisense oligonucleotides translates into biological effects, it is important to not forget that nucleotide therapeutics are large charged molecules also capable of triggering nonantisense effects which can be both sequence specific and nonsequence specific. Sequence-specific effects include potential immune stimulation by sequences such as CpG motifs or G quartets. Charge-related phenomena include thrombocytopenia, which can also be caused by other charged macromolecules such as heparin. Although antisense inhibition seems to be the predominant mechanism responsible for antitumour activity of oligonucleotides, as confirmed in several ongoing clinical trials, it is important to note that additional modes of action may also contribute to the overall responses observed.

How to find an optimal antisense sequence

The first step in validating genes as targets for antisense therapy is to identify those sites on the mRNA which are accessible and do not show sequence homologies with other genes of importance. The rationale behind the design of effective antisense oligonucleotides is based on the notion that mRNA is single-stranded at the AUG site—to allow ribosomal entry—and thus this site should also be accessible for oligonucleotide hybridisation. Oligonucleotides targeting the start codon have been used successfully for several genes, although other sites could prove to be even more effective.[7–9] Various in vitro techniques have been used in order to facilitate selection of target sites for antisense action; many of these techniques are combinatorial approaches based on annealing reactions with arrays of antisense species[10] or assessment of target accessibility by use of RNase H mapping.[11] In addition, computational attempts to predict the secondary structure and folding pattern of mRNAs have been described, and target screening with computer programs such as "mfold" or "RNAstructure" could prove to be a valid strategy for selecting effective antisense sequences.[12] This approach does not take account of other factors that could contribute to antisense efficacy, such as the three dimensional structure in vivo or accessibility of the target site for RNase H, but it substantially reduces the number of

oligonucleotides which have to be tested by high throughput screening. By comparison of the sequences of antisense oligonucleotides of varying effectiveness, the tetranucleotide motif TCCC was identified in 20 of the 42 most effective antisense sequences.[13] Thus, in addition to the techniques described above, the prediction of target sites on the basis of this motif may further assist in the design of antisense therapeutics.

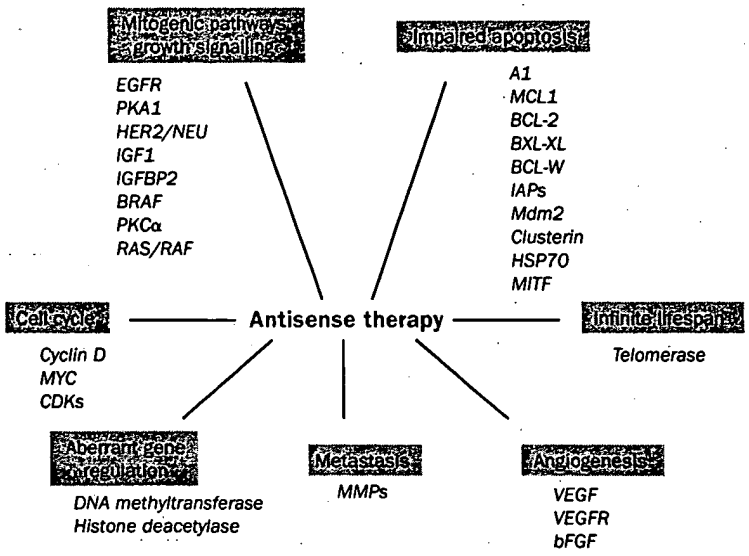

Figure 4. An overview of families of antisense targets.

Use of antisense oligonucleotides in vivo

In initial experiments, the activity of antisense oligonucleotides was limited by the susceptibility of the natural phosphodiester backbone to degradation by cellular nucleases. Several sugar, base, and backbone modifications have been investigated to make these molecules stable enough for clinical use. Significant improvements have been achieved with modifications of the backbone such as replacement of the oxygen atom of the PO moiety by sulphur (phosphorothioates, PS), a methyl group (methylphosphonates), or amines (phosphoramidates). However, although these analogues overcome the stability problem, only the phosphorothioate modification results in antisense compounds that combine serum stability, reasonably high RNA binding affinity, and the ability to elicit RNase H cleavage of the target RNA.[14] Now, after more than a decade of intensive research, phosphorothioate oligonucleotides still represent the most widely used class of antisense compounds, and several of these analogs are currently being tested in clinical trials. Investigations into their biological properties have identified several potentially toxic nonantisense effects, which become apparent at higher concentrations and include complement activation, thrombocytopenia, inhibition of cell-matrix interaction, and reduction of cell proliferation. In safety studies, repeated administration of these compounds to mice revealed several side-effects, especially with phosphorothioate oligonucleotides containing CpG motifs. When surrounded by particular bases, these unmethylated CpG dinucleotides induce immune stimulation, which leads to cytokine release, decreased platelet count, and hepatotoxicity.[15,16] Despite the current belief that phosphorothioate oligonucleotides do not yet represent the optimum antisense strategy, some have shown encouraging results in the clinic.

To improve the safety and effectiveness of phosphorothioate oligonucleotides, modifications have been aimed at increasing their metabolic stability and enhancing affinity for complementary mRNA. With these modifications, the oligonucleotides have a 0·4 °C lower melting temperature per phosphorothioate linkage than native phosphodiester counterparts.[17] As with antibody–antigen interactions, the affinity of antisense oligonucleotides for their target mRNA is a measure of the stability of the nucleic acid hybrid; high affinity represents high gene-repression activity.[6] The activity of phosphorothioate antisense oligonucleotides can be improved by modification of the ribose. Among the different sites in a nucleoside building block, the 2'-position could prove to be the most valuable.[18] 2' modification with an electronegative substituents such as 2'-O-methyl or 2'-O-(2-ethoxy)ethyl (MOE) groups,[6,19] or a 2'-O,4'-C-methylene bridge (locked nucleic acid) [20] confer an RNA-like C3'-endo conformation to the oligonucleotide, which greatly enhances affinity. However, the RNA-like conformation also abrogates the oligonucleotide's ability to activate RNase H. This problem has been addressed by generating mixed-backbone oligonucleotides that only contain the modified nucleotides at the ends, thus leaving a nonmodified DNA gap in the centre, which remains compatible with RNase H activation. Such "second generation" or "advanced chemistry" antisense compounds have favourable physicochemical, biochemical, and pharmacokinetic properties,[6] and therefore may be appropriate for oral or transdermal formulations.[21]

Targets for antisense therapy

With the completion of the first phase of the Human Genome Project, about 30 000 gene sequences and 100 000 mRNAs are now available as tools to validate candidate genes for antisense therapeutic purposes. To find suitable sequences for treating a given disease, genes which are differentially expressed in diseased and normal tissue first need to be identified. Elucidation of the roles of several cancer-related genes in tumour development is a rapidly progressing area of cancer research and has provided a steadily growing list of candidate genes. The multitude of molecular entities and signalling pathways that regulate the life/death decision in cells, and thus represent potential

Table 1. Antisense therapies in phase I–III clinical trials

mRNA target	Drug	Company or investigator	Size/ chemistry	Tumours evaluated	Development phase	Combination treatment?	Website
BCL-2	G3139 (Genasense)	Genta/Aventis	18-mer/PS	Melanoma, MM, CLL, NSCLC	III*	Y	www.genta.com
PKCα	ISIS 3521 (Affinitac)	ISIS/Eli Lilly	20-mer/PS	NSCLC, solid tumours	I–III*	Y/N	www.isispharma.com
c-RAF	ISIS 5132	ISIS	20-mer/PS	Solid tumours	I–II*	Y/N	www.isispharma.com
Ha-RAS	ISIS 2503	ISIS	20-mer/PS	Solid tumours	I–II	N	www.isispharma.com
Clusterin	OGX-011	OncoGeneX	21-mer/AC	Prostate cancer, NSCLC	I–II*	Y	www.oncogenex.ca
PKA-R1-α	GEM 231	Hybridon	18-mer/AC	Solid tumours	I–II*	N	www.hybridon.com
c-MYB	LR/INX-3001	Gewirtz et al	24-mer/PS	CML	I–II*	N	www.uphs.upenn.edu/ hematol/faculty/gewirtz.htm
Ribonucleotide reductase	GTI-2040	Lorus Therapeutics	21-mer/PS	Solid tumours	I–II*	N	www.lorusthera.com
DNA methyltransferase	MG-98	MethylGene	20-mer/AC	Solid tumours	I–II*	Y/N	www.methylgene.com
p53	OL(1)p53	Bishop et al	20-mer/PS	AML, MDS	I	N	
BCR-ABL	BCR-ABL AS	de Fabritiis et al	26-mer/PS	CML	I	N	

MM, multiple myeloma; CLL, chronic lymphatic leukaemia; NSCLC, non-small-cell lung cancer; CML, chronic myelogenous leukaemia; AML, acute myelogenous leukaemia; MDS, myelodysplastic syndrome; PS, phosphorothioate oligonucleotide; AC, advanced chemistry oligonucleotide; Y, yes; N, no. *Initiation of trial, or additional trials expected, in 2002.

targets for molecular intervention strategies including antisense therapy, are shown in figure 3. The most promising targets are those involved in cell proliferation,[22] apoptosis,[23] angiogenesis,[24] and metastasis[25] (figure 4). A comprehensive overview of the antisense oligonucleotides currently under investigation in clinical trials is provided in table 1.

Target sequences are carefully chosen to avoid hybridisation of full-length oligonucleotides to unrelated targets and to ensure they are long enough that uniqueness within the cellular mRNA pool is preserved. Despite these efforts, antisense oligonucleotides were expected to have side-effects resulting from irrelevant cleavage of non-targeted mRNA by low stringency RNase H[5,26] and from silencing of target genes which are not tumour specific. Interestingly, however, preclinical studies and initial clinical tests did not substantiate these concerns. Thus, normal healthy cells seem to tolerate the transient loss of function of genes involved in growth regulation and cytoprotection better than cancer cells, which carry the proapoptotic burden of multiple genetic alterations and genomic instability. Antisense therapeutics that tackle the apoptotic rheostat, interfere with growth signalling pathways, or target tumour microvasculature to inhibit tissue invasion and metastasis, are particularly promising when complemented by conventional anticancer treatments, because their toxicity profiles do not overlap.

BCL-2 family

BCL-2 and BCL-XL are inhibitors of apoptosis with cytoprotective function. Both proteins reside within the mitochondrial membrane where they act by inhibiting adapter molecules needed for the activation of caspases. Recent evidence also suggests a role for BCL-2 in protecting cells from APAF1-independent death. More distant relatives of the family include BAX and the BH3-only proteins, which promote apoptosis by inducing the release of cytochrome C from mitochondria and counteract the protective activity of BCL-2 and BCL-XL by heterodimerisation.[23] Alterations in this balance that favour cell survival may cause proliferative disorders such as cancer. Overexpression of BCL-2 or BCL-XL is indeed common in many types of cancer and believed to contribute to increased resistance to chemotherapy. But although the prognostic value of BCL-2 and BCL-XL overexpression seems to depend on the tumour type, preclinical and clinical data indicate that both proteins may be good targets for antisense therapy. BCL-XL is a survival factor for undifferentiated endothelium cells. So, given the recent finding that BCL-2 overexpression in endothelial and tumour cells results in the upregulation of VEGF expression and increased microvessel density in tumour xenografts, antisense strands targeted to BCL-2 and/or BCL-XL could provide a therapeutic approach that combines proapoptotic and antiangiogenic activity.

In 1997, the first results of a clinical study investigating G3139 (Genasense, table 1)—an 18-mer phosphorothioate oligonucleotide targeted to the BCL-2 translation initiation site—for treatment of non-Hodgkin lymphoma were reported.[27] This phase I, dose-escalation trial included 21 patients[28] and focused on the safety and pharmacokinetics of the drug when administered by subcutaneous infusion. Local inflammation at the infusion site was the most common side-effect; the maximum-tolerated dose was 147·2 mg/m²/day; and the dose-limiting toxic effect was thrombocytopenia. Of the 21 patients, 9 achieved stable disease, 9 had disease progression, two showed partial responses, and one patient had a complete response. However, a reduction in BCL-2 protein by antisense treatment was observed in only about half of the patients.

Drug resistance has been linked to overexpression of BCL-2 in a number of cancer types including melanoma, which is a classic example of a treatment-resistant tumour. In preclinical studies, G3139 decreased the production of BCL-2 protein, enhanced tumour-cell apoptosis, and, in combination with systemically administered dacarbazine (DTIC), led to major tumour responses in mice with human melanoma xenografts.[29] Other BCL-2 antisense oligonucleotides have also shown promise in preclinical studies.[9,30]

In the clinical setting, it was established that G3139 can be safely administered by continuous intravenous infusion in combination with full-dose DTIC in a phase I/II trial in patients with advanced melanoma. This trial also showed that G3139 causes down-regulation of BCL-2 protein in serial biopsy samples from patients with melanoma, and that this biological activity was associated with major clinical responses (figure 5). The entire group of patients, who all had advanced-stage disease, were still alive more than 1 year later.[31] Transient thrombocytopenia limited the antisense dose to 12 mg/kg each day in patients who also received full-dose DTIC treatment. An international, phase III, randomised trial has been initiated in patients with advanced melanoma by use of a 5-day pretreatment regimen of G3139 administered by continuous infusion at a dose of 7 mg/kg each day, followed by DTIC at 1000 mg/m^2. Additional controlled multicentre trials are ongoing in other tumours including multiple myeloma, CLL, and lung cancer (table 1), and have the goal of increasing the effectiveness of experimental treatment strategies.

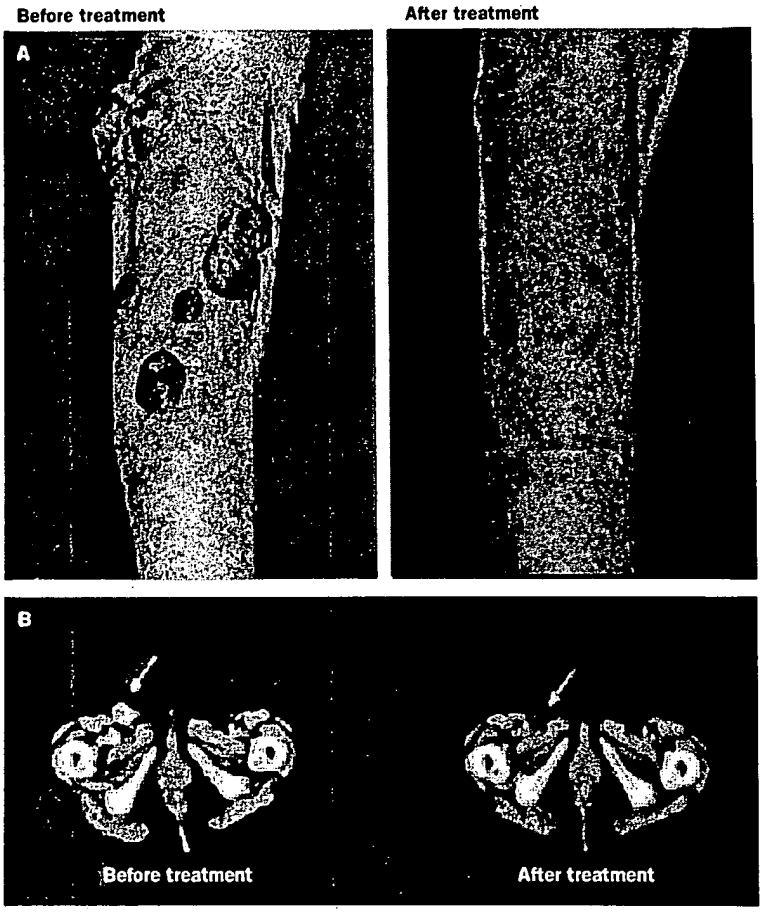

Figure 5. An example of a complete remission of advanced malignant melanoma after chemosensitisation by BCL-2 antisense (Genasense) treatment. Lower right leg (a) and a computed tomography of the pelvis (b) before and after treatment are shown (reference 31).

Alternative splicing of BCL-X pre-mRNA results in two distinct mRNAs: BCL-XL, which codes for the antiapoptotic protein, and BCL-XS, which codes for the proapoptotic variant.[32] Antisense oligonucleotides targeting the BCL-X mRNA or the BCL-XL mRNA specifically have been reported; all these oligonucleotides induce apoptosis in various tumour cells and sensitise tumour cells to chemotherapy.[33–35] Similarly, BCL-X antisense oligonucleotides designed to shift pre-mRNA splicing away from producing the BCL-XL protein to proapoptotic BCL-XS, could also prove effective in sensitising cells to apoptosis.[36] Despite these findings, clinical studies with BCL-XL antisense oligonucleotides have not yet been done. BCL-XL is also expressed in several types of normal tissue including neurons in the brain and certain cells in the bone marrow, which suggests there may be side effects with this approach. Whether BCL-XL antisense therapy can nevertheless prove clinically useful remains to be explored.

Many tumour cells coexpress BCL-2 and BCL-XL and there is uncertainty as to which target is the more important survival factor in a heterogeneous tumour cell population. Although BCL-2 and BCL-XL are functionally similar, there is evidence that they have distinct biological roles in protecting from apoptosis induced by different stimuli. Coupled with the finding that mRNA for BCL-2 and BCL-XL share homology regions with high sequence identity, this information has led to the idea of generating an antisense oligonucleotide which targets both proteins, instead of just one. The design and preclinical testing of a BCL-2/BCL-XL bispecific antisense oligonucleotide was reported recently. This 20-mer, MOE gap-mer oligonucleotide efficiently downreguated BCL-2 expression simultaneously with BCL-XL, and induced apoptosis in tumour cells of diverse histological origins in vitro and in vivo.[30,37]

Protein kinase C

Several studies have examined the role of closely related protein kinase C (PKC) isozymes in various biological

processes. PKC belongs to a class of serine–theronine kinases involved in a myriad of intracellular responses, which are stimulated by activation of G-protein-coupled tyrosine kinases; these kinases are found in both growth-factor-receptor-linked and non-receptor linked forms. Altered expression of PKC has been implicated in the deregulation of cell growth and tumour development.[38] Various inhibitors and modulators of PKC activity have been tested. The clinical limitation of these compounds is their inability to discriminate between the various isozymes of PKC, which may result in unacceptable toxic effects in normal tissues. Several antisense oligonucleotides that specifically target individual members of the PKC family have been studied. On the basis of the activity and safety profile of a murine PKCα-specific antisense inhibitor in mice, a 20-mer phosphorothioate oligonucleotide targeting human PKCα (ISIS 3521) was developed. ISIS 3521 was found to inhibit the growth of glioblastoma xenografts and extended survival in tumour-bearing mice.[39] The biological evidence implicating PKC in the pathogenesis of solid tumours and the activity of ISIS 3521 against human tumour xenografts in mice,[40] led to the initiation of clinical studies with this drug.

The phase I trials of ISIS 3521 were dose-escalation studies in patients with treatment-resistant solid tumours. The oligonucleotide therapy was well tolerated and therapeutic benefit was noted in some cases. In further trials, responses were seen at several different doses of ISIS 3521. Two patients with lymphoma experienced complete responses (after 18 and 9 months of treatment) and neither had recurrant disease after 21 and 14 months, respectively.[41] In one of the phase I trials, ISIS 3521 was administered to 21 patients with end-stage malignant disease for 3 weeks via continuous intravenous infusion, followed by a 1-week treatment-free period. Dose-limiting thrombocytopenia and fatigue were encountered at doses of 3·0 mg/kg per day. The maximum tolerated dose was found to be 2·0 mg/kg/day, which corresponded with doses showing antitumour activity in xenograft models. There was evidence of tumour responses lasting up to 11 months in 3 of 4 patients with ovarian cancer. Continuous intravenous infusion of ISIS 3521 over 3 weeks has so far not shown objective clinical benefit in patients with recurrent high-grade astrocytomas.[42] Median time to progression was 35 days and median survival was 93 days; any toxic effects were mild and reversible.

On the basis of extensive data on ISIS 3521 as a single-agent, a phase I/II study combining the PKC antisense oligonucleotide with carboplatin and paclitaxel in patients with stage IIIB or IV non-small-cell lung cancer (NSCLC) was initiated.[43,44] A recent update of 24 evaluable patients with NSCLC showed that 1 year survival was 78 %, with a median survival of 18 months. Survival in comparable patient cohorts receiving chemotherapy alone was reported to be 8 months. The combination of ISIS 3521, carboplatin, and paclitaxel was well tolerated with manageable thrombocytopenia and neutropenia as the main side-effects. These promising results led to a 600-patient randomised phase III clinical trial of ISIS 3521 in combination with chemotherapy for NSCLC, which is now underway.[45]

RAF kinas

The *RAF* proto-oncogene encodes a serine–threonine kinase that is activated by the RAS protein as part of the mitogen-activated protein kinase (MAPK) signalling cascade.[46] c-RAF kinase binds to BCL-2 and thus may also play an indirect role in the regulation of apoptosis. Mutations of the *RAS* or *RAF* gene resulting in their constitutive activation have been identified and their aberrant expression reported in many tumours where it may serve as a negative prognostic factor. Because 75–90% of all pancreatic adenocarcinomas harbour activating mutations in the *k-RAS* oncogene it has been expected for a long time that inhibitors of the RAF-1-MEK-MAPK pathway may be useful to control diseases associated with abnormal cell proliferation. A series of 34 phosphorothioate antisense oligonucleotides were designed and screened for reducing c-RAF mRNA concentrations in vitro.[8,47] ISIS 5132, a 20-mer targeting a site at the 3' untranslated region, was identified as the most potent in inhibiting c-RAF expression, and showed antiproliferative and antitumour activity against various tumour-cell lines in vitro and in xenograft models in mice.[8]

2 h intravenous infusions of ISIS 5132 were administered 3 times a week for 3 consecutive weeks with doses ranging from 0·5 to 6·0 mg/kg; the infusions were well tolerated without dose-limiting toxic effects.[48] Furthermore, an additional phase I dose-escalation trial (continuous intravenous infusion of ISIS 5132 for 21 days every 4 weeks) in 34 patients suffering from different refractory solid tumours was done.[49] Continuous administration of ISIS 5132 up to 4·0 mg/kg led to no dose-limiting toxic effects. At 5·0 mg/kg, fever and thrombocytopenia were dose-limiting. One patient who had treatment-refractory ovarian cancer experienced a reduction in CA125 concentration of more than 90%; 2 other patients achieved stable disease for 9 and 10 months. Statistically significant downregulation of c-RAF1 mRNA by ISIS 5132 in peripheral blood mononuclear cells of patients with advanced malignant disease was observed in most cases.

Accumulation of evidence for proof of principle and the confirmation of an acceptable safety profile, led to the initiation of several phase II trials of ISIS 5132. However, in one study investigating the antitumour activity of ISIS 5132 (4 mg/kg per day, 21-day continuous intravenous infusion every 4 weeks) in 22 pretreated patients with recurrent ovarian cancer, the drug did not seem to have therapeutic activity as a single agent.[50] Results of other phase II clinical studies targeting c-RAF kinase in prostate and colon cancer are pending.

Protein kinase A

The cAMP-dependent protein kinase A (PKA) is involved in various biological functions including cell proliferation, induction of gene expression, and ion-channel regulation. PKA is composed of two catalytic and two regulatory subunits and has type–I and type-II isozymes containing different R subunits, termed RI and RII. Of the 4 isoforms of R increased expression of RIα is associated with cell proliferation and neoplastic transformation. Accordingly, overexpression of the RIα subunit of PKA is found in many

tumours in which it is a negative prognostic factor.[51] The catalytic subunit of PKA also phosphorylates the EGF receptor, which is accompanied by decreased auto-phosphorylation and diminished tyrosine kinase activity. The finding that decreased expression of RIα and thus release of the catalytic subunit correlates with growth inhibition induced by cAMP analogues in transformed cell lines, has prompted studies to investigate the potential of antisense inhibitors of RIα as anticancer agents that interfere with the mitogen-activated signalling pathway. Oligonucleotides targeted to various sites within the coding region of the RIα mRNA that showed antiproliferative activity in different tumour cells and cooperatively acted with EGF-receptor inhibitors were identified.[52]

The 18-mer mixed-backbone antisense oligonucleotide GEM231 (table 1), designed to interfere with the production of the RIα regulatory subunit of PKA, inhibited the growth of tumour cell lines in vitro and displayed antitumour activity against human tumour xenografts when given orally to mice.[21,53] Phase I preliminary data show that escalating doses of GEM231 were also well tolerated in repeat cycles when administered twice weekly by intravenous injections at doses of up to 360 mg/m² (equivalent to about 7–9 mg/kg). Additional studies designed to evaluate the safety and efficacy of GEM231 in combination with taxotere or taxol in patients with advanced cancers have been initiated. Early results suggest that side-effects caused by GEM231 were mild and reversible and do not overlap with the side-effects caused by taxanes.

p53/MDM2

The tumour suppressor p53 is a master-switch for cell cycle regulation and apoptosis. Terms like "guardian of the genome" have been coined for p53 and mutations resulting in its inactivation or deletion have been found in a number of hematological and non-hematological malignant diseases. OL(1)p53 (table 1) is a phosphorothioate antisense oligonucleotide complementary to 20 bases within exon 10 of the p53 mRNA. A total of 21 patients with either refractory acute myelogenous leukemia (AML) or advanced myelodysplastic syndrome received OL(1)p53 at doses up to 0·25 mg/kg/h for 10 days by continuous infusion during two initial phase I trials. Neither specific toxic effects nor complete hematological responses were observed. More recently, 9 patients participated in a phase I study evaluating OL(1)p53 as a bone-marrow purging agent [54]. Harvested marrow was incubated with OL(1)p53 at 10 μM of the oligonucleotide for 36 h before transplantation. However, clear clinical benefits were not observed.

MDM2 is a negative feedback regulator of p53 which interferes with the control of cell proliferation and apoptosis. It interacts not only with p53, but also with p14ARF, which antagonises its function as a suppressor of p53. MDM2 is amplified/overexpressed in various tumours. A mixed-backbone antisense oligonucleotide targeting MDM2 released p53, which resulted in increased p21/WAF1 expression. This effect was synergystic with different classes of anticancer agents and significantly increased apoptosis in tumour cells in vitro. In mice, MDM2 antisense drugs have shown antitumour activity against human tumour xenografts and seem to potentiate the effect of anticancer agents.[55]

c-MYB

The *c-MYB* proto-oncogene encodes a DNA-binding transcription factor involved in the control of the G1/S transition of the cell cycle.[56] c-MYB is located on chromosome 6q22–24, on which abnormalities have been observed in cells from leukaemias, lymphomas, colon carcinomas, and malignant melanoma.[57] Ratajczak and colleagues[58] used 18-mer and 24-mer phosphorothioate antisense oligonucleotides targeted to codons 2–7 and 2–9, respectively, of the c-myb gene to inhibit the proliferation in AML, CML, and T-cell leukaemia cultures by induction of apoptosis. In a clinical pilot study, the 24-mer antisense oligonucleotide LR-3001 showed promise in purging ex vivo bone-marrow harvests from patients with CML before autologous transplantation. The patients (seven in chronic phase CML and one in accelerated phase) received chemotherapy with busulfan and cytoxan, followed by re-infusion of previously cryopreserved mononuclear cells purged for 24 hours with LR-3001. Seven of eight patients were reconstituted successfully. In 4 of 6 evaluable patients, 85–100% normal metaphases were found 3 months after engraftment, supporting the notion that significant ex vivo purging of the marrow graft was achieved. A phase II study has been initiated to validate these promising findings.

DNA methyltransferase

Aberrant expression of the DNA methyltransferase enzyme is implicated in multiple tumorigenic pathways and there are several lines of evidence suggesting that this alteration plays a causal role in neoplastic transformation and tumour development. The possible mechanism by which overexpression of DNA methyltransferase may induce tumorigenesis is promoter hypermethylation, which inactivates a large number of genes that induce apoptosis, and suppress tumorigenesis, tumour invasion, and angiogenesis, or that control DNA replication and cell-cycle progression. Inhibition of DNA methyltransferase by antisense treatment was shown to revert the malignant phenotype of tumour cells in vitro and in mice.[59,60] The mixed-backbone antisense oligonucleotide MG-98, originally developed by Hybridon Inc and further evaluated by MethylGene Inc (table 1) was shown to effectively downmodulate the methylation of candidate genes involved in tumorigenesis and has found its way to early clinical testing. MG-98 was given to 9 patients with advanced solid tumours as a continuous 21-day intravenous infusion, with cycles to be repeated at 4-week intervals. Dose-limiting, drug-related increases in the production of transaminases were found in 2 of 2 patients receiving a dose of 240 mg/m² per day. Reductions of DNA methyltransferase mRNA considered to be biologically relevant were seen at the lowest dose (40 mg/m² per day). However, changes in DNA methyltransferase mRNA did not translate into clinical benefit.[61]

BCR-ABL

The Philadelphia chromosome is the result of a translocation found in chronic myelogenous leukemia (CML) cells resulting in the juxtaposition of the *ABL* gene (normally found on chromosome 9) and the *BCR* gene of chromosome 22. The resultant p210 fusion protein functions as a tyrosine kinase and is believed to cause the neoplastic phenotype of CML. A 26-mer oligonucleotide targeted against the BCR-ABL transcript has shown selective inhibition of in vitro cell growth. This strategy also proved successful in an SCID mouse model. Mice with CML survived 18 to 23 weeks, whereas controls died within 8–13 weeks after tumour cell inoculation.[62] On the basis of these encouraging preclinical findings, clinical studies investigating ex vivo bone-marrow purging by BCR-ABL antisense oligonucleotides in patients with CML were initiated.[63] The bone-marrow sections from eight patients were incubated for 24 or 72 hours with 150 μg/ml BCR-ABL antisense oligonucleotides. Two complete karyotypic responses were noted; the karyotypes of the remaining 6 patients showed no or only minimal responses. These results translated into a persistent second chronic phase lasting 14–26 months after transplantation in three patients.

Clusterin

Clusterin, also known as testosterone-repressed message 2 (TRPM2) or sulfated glycoprotein 2, has been implicated in a variety of physiological and pathological processes. An increasing body of evidence supports a role for this protein in apoptotic cell death. More recent studies also suggest that clusterin acts in a chaperone-like way—similar to that of small heat shock proteins—potentially inhibiting stress-induced protein misfolding in vitro; it also improves cell survival in vivo.[64] In prostate cancer, experimental and clinical studies provided evidence that clusterin concentrations increase in response to therapeutic cell death signals, such as androgen ablation or chemotherapy, and protect against apoptosis.[65] When overexpressed, clusterin confers a hormone-resistant or chemotherapy-resistant phenotype in a number of malignant diseases including prostate cancer, indicating that it is an attractive target for antisense therapy. Phase I/II clinical trials evaluating the therapeutic potential of OGX-011, a 21-mer MOE gap-mer oligonucleotide targeting the translation initiation site of clusterin, in combination with androgen ablation or chemotherapy have been initiated in patients with prostate cancer and will be initiated in the near future in patients with other types of solid tumour including NSCLC (table 1).

Inhibitors of apoptosis proteins

The inhibitors of apoptosis proteins (IAPs) act as roadblocks in the apoptosis signalling pathway by directly binding to caspases, thereby blocking their processing and activity. Evolutionarily conserved from viruses to mammalian cells, these proteins are characterised by a 70 aminoacid domain called baculoviral inhibitory repeat (BIR). The IAPs XIAP, c-IAP1, c-IAP2 can interact with the effector caspases 3 and 7, but also with the initiator caspase 9. The IAP survivin seems to associate with caspase 3 in the vicinity of centrosomes during mitosis and is required for suppression of caspase-mediated- cleavage of centrosome-associated p21waf1.[66] Recent evidence suggests that survivin also interacts with pro-caspase 9 and that phosphorylation of survivin by $p34^{cdc2}$–cyclin B1 is required for its cytoprotective function. Survivin is expressed in the G2/M phase and its overexpression in cells may overcome the G2/M checkpoint to enforce progression of cells through mitosis. In colorectal, gastric, breast, bladder, and lung cancers, and in certain leukaemias and lymphomas, survivin expression is associated with shorter survival and correlates with a higher stage of disease. Together with the finding that survivin lacks expression in terminally differentiated tissues, but becomes re-expressed during neoplastic transformation, this IAP deserves attention as a truly tumour-specific target for antisense therapy. Antisense oligonucleotides targeting different sites within the survivin mRNA have been tested in different cell systems and shown to induce apoptosis directly or sensitise cells to additional apoptotic stimuli.[67]

Other potential targets for antisense therapy

As a consequence of the encouraging preclinical findings with evidence of selective inhibition of Ha-RAS expression by ISIS 2503, a 20-mer phosphorothioate antisense oligonucleotide targeting the translation initiation site of the Ha-RAS mRNA, ISIS 2503 (table 1) has been evaluated alone and in combination with chemotherapy in phase I and II trials in a number of solid tumours.[68] There is clear evidence that ISIS 2503 is well tolerated. However, ISIS 2503 has not shown antitumour activity as a single agent in the cancer types so far tested and antisense strategies targeting other RAS isoforms seem more attractive, since 75–90% of pancreatic adenocarcinomas and 50% of colon cancers harbor activating mutations in K-RAS but not Ha-RAS.[69] Antisense oligonucleotides targeting the ribonuclease reductase (table 1), an enzyme of importance in DNA synthesis and cell proliferation, have recently reached the stage of early clinical evaluation. Insulin-like growth factor 1 (IGF1) has mitogenic and antiapoptotic effects modulated by insulin-like growth-factor binding proteins (IGFBPs) in various tissues. Downregulation of IGFBP5 expression by antisense oligonucleotides inhibited the growth of Shionogi cancer cells in vitro and inhibited the growth of androgen-independent tumours after castration.[70] This finding suggests that the increase of IGFBP5 concentrations observed after castration in models of prostate cancer is an adaptive cell-survival mechanism that helps potentiate the antiapoptotic and mitogenic effects of IGF1, thereby accelerating androgen-independent progression towards a treatment resistant phenotype.[71] Clinical studies with antisense oligonucleotide (OGX-133) designed to attenuate IGFBP5 function in patients with advanced prostate cancer are being planned.

The cellular Fas-associated death domain (FADD)-like interleukin-1β-converting enzyme-like (FLICE) inhibitory protein (cFLIP) resembles caspase 8 in structure, but lacks protease activity. It interacts with both FADD and caspase 8 to inhibit the apoptotic signal of death receptors and, at the same time, can stimulate survival signalling through

Search strategy and selection criteria

Published data for this review were identified by searches of MEDLINE, EMBASE, CancerLit, METACRAWLER, the websites of companies that produce antisense compounds (table 1), and references from relevant articles up to August 2002. The terms "cancer therapy", "antisense", "oligonucleotides", "RNA", "gene expression/regulation", "oncogenes", "apoptosis", "angiogenesis", "metastasis". Only articles published in English were used. In addition, we contacted researchers, clinicians and companies working on antisense strategies.

activation of NFκB. Inhibition of FLIP may also be useful in the treatment of carcinomas with acquired resistance to death-receptor-induced apoptosis and chemoresistance.[72] The stress-inducible heat-shock protein Hsp70 is a chaperone selectively expressed in tumours and tumour cell lines, which seem to depend on its constitutive high expression to suppress a transformation-associated death programme. Depletion of Hsp70 by antisense constructs was shown to induce cell death; the mechanism of action was possibly disruption of Hsp70-BCL-2 interaction, but Hsp70 has recently also been shown to bind and inhibit Apaf-1 and apoptosis-inducing factor. Interestingly, cell death was induced in various tumour cell lines and in cells from primary tumours, but not in normal epithelial cells and fibroblasts.[73] Whether Hsp70 indeed confers a survival advantage selectively to tumour cells and whether targeted disruption of *Hsp70* gene expression can provide a reasonable therapeutic index remains to be determined. Further preclinical data are clearly needed to convincingly demonstrate that chaperoning by Hsp70 is more dispensable for the function of normal tissues than for the survival of tumours. Other genes currently validated as targets for antisense therapy in preclinical studies include growth-factor-receptor tyrosine kinases such as HER2/neu, the epidermal and vascular endothelial growth-factor receptors, transcription factors involved in cell survival like NFκB, the myriad of protein kinases involved in cell cycle regulation, and thymidylate synthase. Additional attractive targets of great potential and importance for antisense strategies highlighted by most recent reports include BRAF, mutated in about 60% of melanomas and at somewhat lower frequencies in a variety of other malignant diseases,[74] and MITF, a transcription factor shown to regulate BCL-2 in cells of the melanocytic lineage as well as in certain bone cells.[75] Although the available data are still preliminary, there is hope that some of these cancer-related targets will maintain their allure beyond clinical scrutinisation.

The future of antisense

There is increasing evidence that antisense oligonucleotides can work in a sequence-specific manner in patients and will finally live up to their promise. Although a number of challenges remain, these are probably easier to overcome in oncology than in any other field since there are large unmet medical needs. Furthermore, several antisense oligonucleotides have displayed acceptable toxicity profiles. Antisense strategies which aim at restoration of apoptosis signalling, alter signalling pathways involved in cell proliferation, or target the tumour's micro-vasculature, may prove particularly useful in combination with conventional anticancer agents. Lowering the apoptotic threshold of cancer cells could prove to be the single most attractive strategy to overcome chemo- and radiation resistance. But studies focusing on other possible mechanisms of action for antisense oligonucleotides, beyond anticipated clinical successes, will be instrumental to progress in the field. These studies are mandatory to highlight antisense oligonucleotides as rationally designed molecules with predictable properties; the potential for rapid development and improvement places them apart from conventional drugs.

Conflict of Interest

BJ and his Vienna Laboratory received limited financial support or antisense compounds free of charge from ISIS Pharmaceuticals, Genta Inc, OncoGeneX Technologies Inc, and Hybridon Inc. BJ is currently on sabbatical from all academic positions at the University of Vienna and serves at OncoGenex Technologies, Inc as Vice President, Clinical Development. The group of UZW received limited financial support and antisense compounds free of charge from Novartis Pharma AG.

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Clinical picture

Subcutaneous seeding of pancreatic carcinoma along a VP shunt catheter

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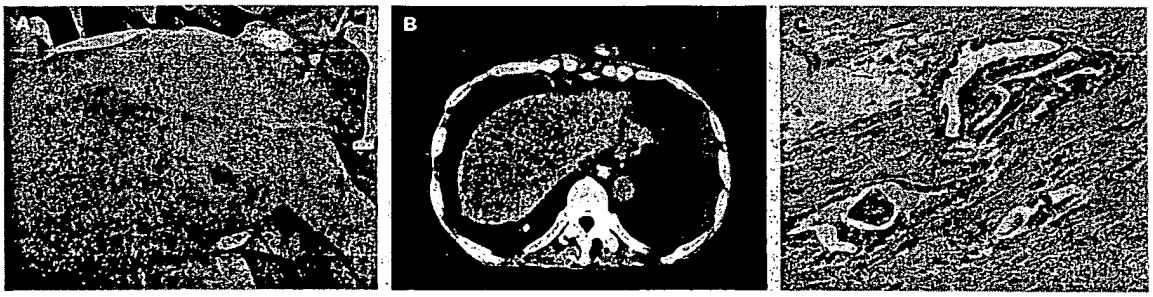

A 61-year-old woman presented with subarachnoid haemorrhage 3 years ago and had a ventriculoperitoneal (VP) shunt inserted to treat normal-pressure hydrocephalus, after which she regained normal daily activity. She later presented with a subcutaneous painless mass along a VP-shunt catheter (figure a). A computed tomography scan of her abdomen showed ascites, free air, and an isodensity mass around the catheter on the anterior abdominal wall (figure b). She underwent an emergency exploratory laparotomy and was subsequently diagnosed with a pancreatic carcinoma. Histopathological examination of the skin around the catheter showed a small number of neoplastic glands in the fibrotic background (figure c). These features are consistent with metastatic adenocarcinoma of pancreatic origin. It is well known that a VP shunt as an artificial link between vessels can facilitate the spread of brain tumour cells in the cerebrospinal fluid. Similarly, iatrogenic spread of malignancy may occur along a VP shunt catheter. This is the first case of VP-shunt-related skin metastasis of aggressive pancreatic carcinoma.

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